Secondary Metabolite Profile in Mature and Old Leaves of Four Piper Species

by Michael Raharja Gani

A. SELECT PST SECTION

8. Pharmacology/Ethnomedicine/Phytochemistry

B. SELECT ARTICLE TYPE

Research article

C. SELECT SECTION

Regular issue

Secondary Metabolite Profile in Mature and Old Leaves of Four Piper Species

Abstract

Piper species is a potential medicinal plant, empirically known for its effectiveness in curing various diseases, particularly in Indonesia. Therefore, this research aimed to evaluate the similarities and differences in the profile of secondary metabolite compounds in mature and old leaves of four *Piper* species using Gas Chromatography-Mass Spectrometry (GC-MS). The analysis was also carried out to identify the specific compounds found in each species at different leaves development stages. Samples used were mature and old leaves from four species of *Piper*, namely forest betel (*Piper aduncum* L.) (PA), red betel (*Piper crocatum* Ruiz & Pav.) (PC), Javanese chili betel (*Piper retrofractum* Vahl.) (PR), green betel (*Piper betle* L.) (PB). Subsequently, samples were extracted using ethanol solvent and secondary metabolite profile was detected through GC-MS. A total of 40 secondary metabolite compounds were found in mature and old leaves of four species. The results showed that alkaloid content contributed 25% of the total compounds detected, while fatty acids yielded the largest portion 27.5%. Based on PCA score plot analysis, a significant grouping of secondary metabolite compounds was observed in all species, where PC was categorized separately on the right, and the other species were on the left. Several specific compounds were also found only in one species and not in others. Similar to mature and old leaves, some compounds were discovered in one of the developmental phases. In conclusion, this research showed that each *Piper* species had a distinct compound profile specific to each other in both mature and old leaves.

Keywords: profile, secondary metabolite, mature leaves, old leaves, *Piper*, GC-MS

Introduction

The piper plant is used for many different things all around the world. It can be used in herbal medicines, culinary applications, decorative displays, and customary rites. It has substantial botanical diversity, with about 700 species recognized globally (1) and an estimated 1400-2000 variants arising from various nations. Only Java Island in Indonesia is home to about 23 different species of Piper known to science. Most species are found to be able to survive at elevations between sea level and 2500 meters, while very few are able to reach elevations over 3000 meters (2). In particular, Indonesia uses the betel plant in traditional medicine. The well-known effects of the green betel (*Piper betle* L.) as an antidiabetic, immunomodulator, platelet inhibitor, antioxidant, and anticancer agent make it stand out (3). The Javanese chili is another noteworthy Piper species that may be found in the area

PC originated in Peru and has since spread to several countries, including Indonesia. This plant is characterized by its bushy growth pattern, with tendrils and segments stems, and a node spacing of between 5-10 cm, where the root grows. Leaves are elliptical, acuminatus, sub-acute at the base with a tapered top, flat edges, shiny or hairless, with a length of 9-12 cm and width of 4-5 cm.

Pinnatus leave veins are found in the lower half, comprising 4-5 pairs, forming a bullulatus-lacunosa pattern. The petiolus is 10 mm long, with spikes ranging from 90 to 110 mm long, and 5 mm thick. The top leaves display a dark green with silver markings along the veins, while the lower sections exhibit a purple coloration, having a slightly slimy texture and a bitter taste, along with a less distinct aroma (7).

Based on its morphology, the Javanese long pepper is a climbing plant, characterized by round leaves, lanceolate, and wide with green to dark green. The fruit has varying shapes and sizes ranging from small lengths (cylindrical), flat (filiform), elliptical (conical), and short round (globular). The stem is round and large, has a diameter of ± 5-7 cm, length of the main stem is 2.93-9.82 cm, with significantly varying color from black, and brown to blackish brown. The morphology of PB is a perennial dioecious climber, with large leaves, 15-20 cm long, broadly ovate, slightly cordate, shortly acuminate, acute, entire, glabrous, yellowish or bright green, shining on both sides (8).

Piperaceae species are widely recognized for the rich content of essential oils, alkaloids, and phenols (9). Alkaloids and phenols, which are common components of the Piperaceae family, are significant physiological ingredients that aid in controlling plant growth and providing defense against diseases and insect pests (10). These compounds have also been extensively used as natural plant products, mainly applied in the pharmaceutical and food industries (11–13). Currently, the research of plant metabolomic has gained significant attention due to its potential for diagnosing metabolite changes in low molecular weight metabolite and the underlying biochemical mechanisms (14). This shows that the use of plant metabolomic research to investigate the distribution and profile of secondary metabolite in plant organs is an important step in identifying the medicinal properties of various plant parts.

Based on previous investigations, there is no research that has explored and comprehensively compared secondary metabolite profile in mature and old leaves of four *Piper* species, namely PA, PC, PR, and PB. Consequently, this research aimed to evaluate the similarities and differences of secondary metabolite profile in mature and old leaves of four *Piper* species using GC-MS. The specific compounds found in each species at different ages of leaves were also explored.

Materials and Methods

Chemical and Plant Materials

Chemical for extraction was carried out using Merck Sigma Aldrich absolute ethanol and liquid nitrogen. Samples used were mature (M) and old leaves (O) from PA, PC, PR, and PB, with three replications for each age and species. Leaves samples were collected from Sleman Regency, Yogyakarta Special Province, Indonesia (Longitude: 110.363416, Latitude: -7.669998).

Metabolite Profile

Extraction of Secondary Metabolite

Sample extraction used the maceration method reported in previous research (15) with modifications. A sample of 20 g leaves was added with liquid nitrogen and crushed using a mortar to obtain a powder form. Subsequently, the powder was transferred to an Erlenmeyer, 15 ml ethanol was added, and stirred. Samples were incubated at room temperature for 72 hours, filtered, and the extract was evaporated in a petri dish for analysis using GC-MS.

GC-MS Analysis

GC-MS analysis was conducted using Shimadzu GCMS-QP2010S equipped with an Agilent DB-5MS column. The column specifications included a length of 30 m, 0.25 mm ID, 0.25 um film, Helium carrier gas, and EI 70Ev ionizer. GC-2010 specifications were column oven temperature of 70.0°C, injection temperature of 300.00°C, splitless injection mode, sampling time of 1.00 min, flow control mode pressure, pressure 30.0 kPa, total flow 35.6 mL /min, column flow 1.65 mL/min, linear velocity 29.6 cm/sec, purge flow 3.0 mL/min, split ratio 49. Furthermore, the ion source temperature was 250°C, the interface temperature of 305°C, the solvent cut time was 5 minutes, and the detector gain relative mode. Total GC running time was 80 minutes and the relative percentage amount of each component was calculated by comparing its average peak area to the total areas.

Data Processing and Statistics

The data from GC-MS analysis was summarized based on the Similarity Index (SI) value > 80%. Compound names, chemical formulas, and Retention Time values were tabulated and analyzed with the Metaboanalyst program (https://www.metaboanalyst.ca/MetaboAnalyst/). The distribution and sample grouping were visualized using principal component analysis (PCA), an unsupervised approach that lowered the dimension of the data sets. The threshold for identifying possible outliers in the dataset was set at a 95% confidence interval in the PCA score plot.

Results and Discussion

Metabolomic Profile of Samples

The results of GC-MS analysis showed that 40 secondary metabolite compounds were detected in mature and old leaves of all *Piper* species. These compounds were divided into 12 groups, namely monoterpenes (5%), sesquiterpenoids (15%), terpenes (2.5%), fatty acids (25%), alkanes (22.5%), phytosterols (2.5%), benzene (5%), terpenoids (5%), phenols (2.5%), phenylpropanoids (2.5%), tocopherols (5%), and azacycloalkalene (2.5%), as presented in Figure 1. *Piper* species has been widely explored and the phytochemical investigations globally led to the isolation of several physiologically active compounds, including alkaloids, amides, propenyl phenols, lignanes, neolignanes, terpenes, steroids, kawapyrones, piperloids, chalcons, dihydrochalcones, and flavones. Biological activities of different species have special emphasis, indicating a wide spectrum of pharmacological activities (16). Furthermore, the 40 compounds detected were analyzed with Metaboanalyst to determine the grouping of specific compounds in each species with different ages of leaves.

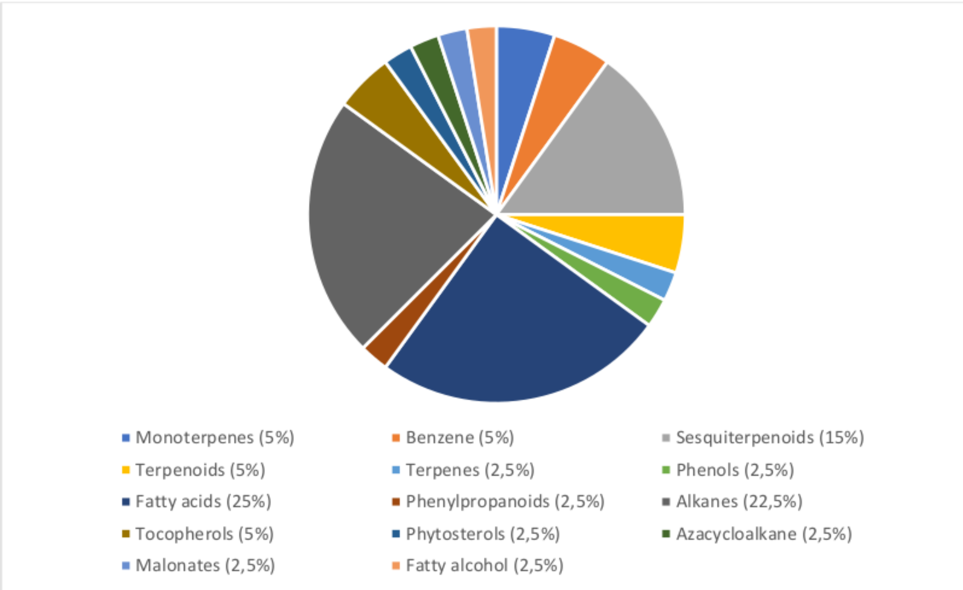

Figure 1. Classification of 40 secondary metabolite compounds in mature and old leaves of four *Piper* species.

Grouping Compounds

In order to make it easier to see the similarities and differences across the data sets, PCA was utilized to categorize metabolite phenotypes and find the differential metabolite. Every point of a PCA score represented a single sample, and the score plot displayed the distribution of samples. This demonstrated that whilst distinct metabolomic activities were distributed, similar metabolomic processes were clustered (17). As seen in Figure 2., PCA was used to find variations in metabolite profiles across eight datasets that included two leaf development stages and four Piper species.

In this research, PCA was carried out on 40 secondary metabolite compounds resulting from GC-MS analysis. The score plot showed that samples from the same *Piper* species were closely clustered, while those from different species were separated from each other. PCA results of all detected compounds identified four groups, where secondary metabolite compounds in PC were grouped separately on the right. Meanwhile, PA, PR, and PB were categorized on the left, as presented in Figure 2. PC1 and PC2 accounted for 29% and 17.5% of the variance in the data, respectively, showing specific secondary metabolite compounds found in each *Piper* species. However, there were no differences in the grouping of compounds in mature and old leaves of each species.

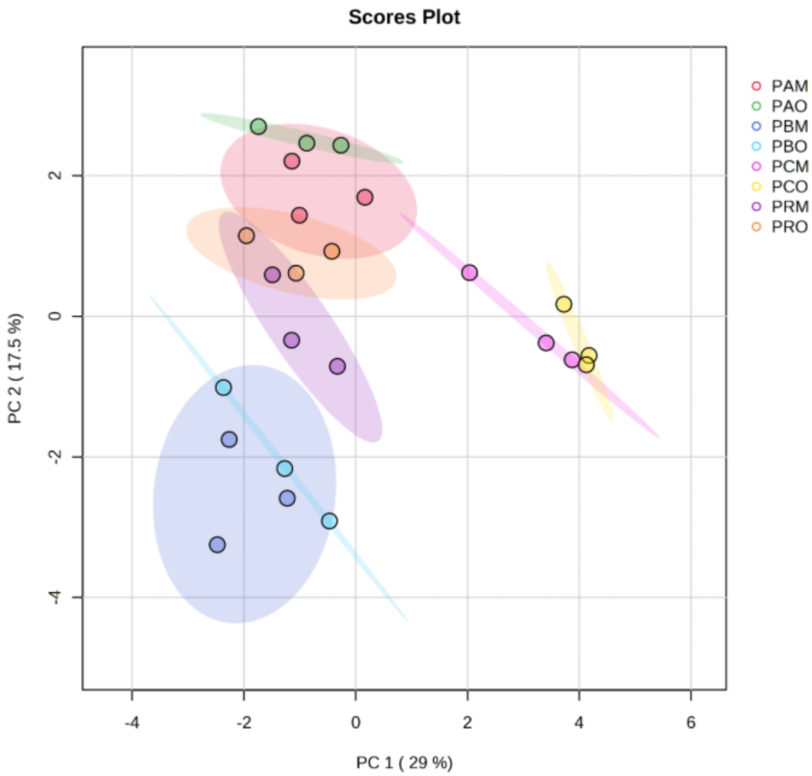

Figure 2. PCA score plot of PAM, PAO, PBM, PBO, PCM, PCO, PRM, PRO.

The results of PCA analysis showed that PC had a different group of compounds compared to other species. Morphologically, PC had the most different characters compared to others, namely in the morphological characters of leaves. The upper leaves are dark green, with a silvery appearance around the veins, while the lower parts are purple (7). These leaves are slimy and have a bitter taste with a less specific odor compared to other species. PC leaves are found to include flavonoids, polyphenolic chemicals, tannins, and essential oils, according to chromatography analysis (5).

Heatmap analysis was carried out on the 40 detected compounds to determine the grouping and profile of specific compounds in each sample, as shown in Figure 3. Based on heatmap analysis, the profile of secondary metabolite specific compounds for each species was identified at different ages. In PC, specific compounds were found in mature leaves (PCM), namely Benzofuran, 2,3-dihydro (benzene), Myrcene (Monoterpenes), and 1,6,10-Dodecatrien (Terpenoids), while old leaves (PCO) contained Trans-Ocimene (Monoterpenes), and alpha Bisabolol (sesquiterpenes). The extract from PC leaves contained alkaloids, carbohydrates, water, tannins, phenols, flavonoids, and essential oils, per earlier study (5). According to the results of (18), certain compounds were found in PC leaves, such as flavonoids with groups like quercetin and aurone, and essential oils with monoterpene components like α-thujene, α-pinene, sabinene, ß-myrcene, α-terpinene, ß-phellandrene, γ-terpinene, α-terpineol, terpinolene, and copaene. Further present were neo-lignans such as 1-allyl-3,5-dimethoxy7-methyl-oxo-6-(3,4,5-trimethoxyphenyl) bicyclo[3,2,1]oct-2-en-8-yl acetate and sesquiterpenes such as caryophyllene, α-caryophyllene, and germacrene D. Additionally detected were compounds classified as alkaloids, tannins-polyphenols, steroids-terpenoids, and saponins. PC leaves have been shown to have anti-inflammatory, antibacterial, antifungal, antihyperglycemic, and anti-proliferative qualities in a number of investigations into its pharmacological characteristics.

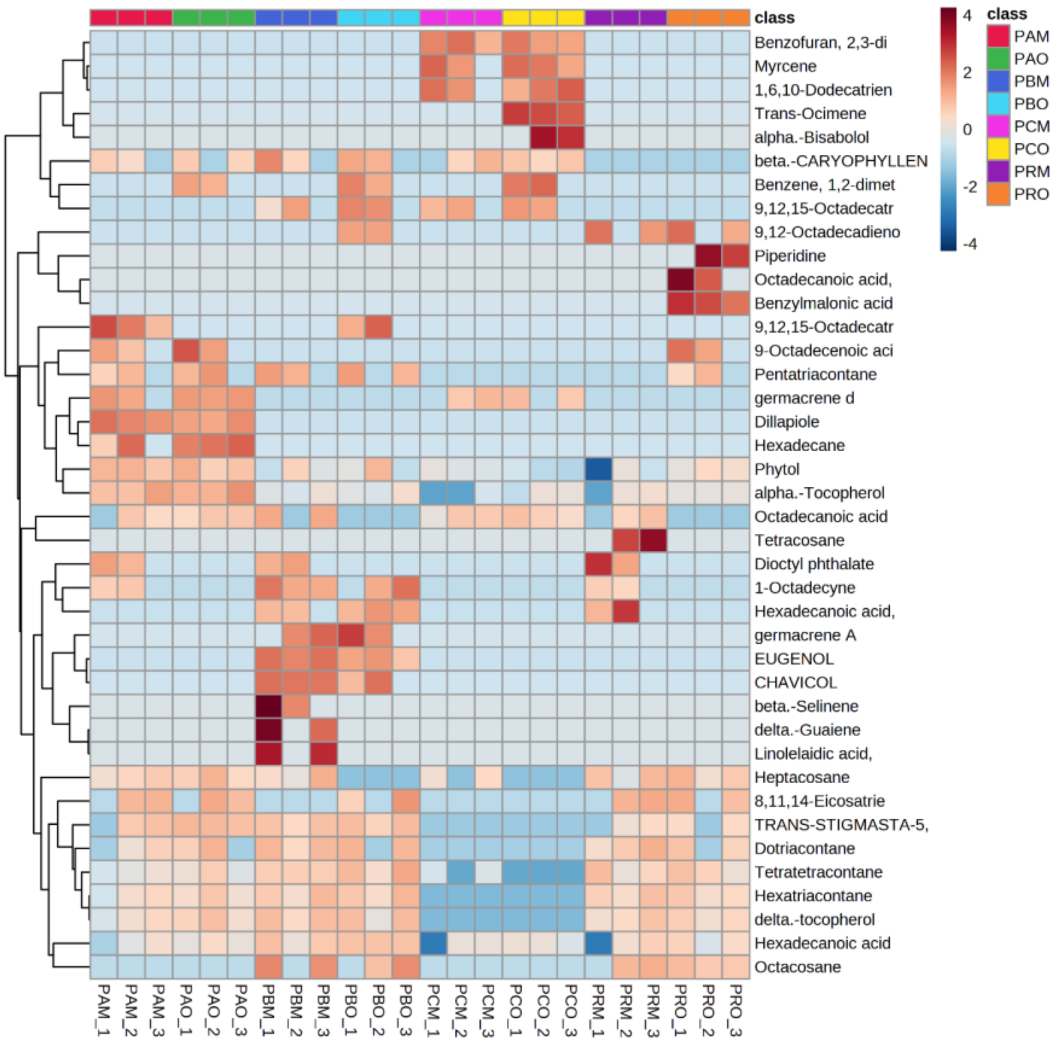

Figure 3. Heatmap clustering among PRO, PRM, PCO, PCM, PBO, PBM, PAO, and PAM groups.

In PR, specific compounds were detected in mature (PRM) and old leaves (PRO), including Piperidine (azacycloalkane), Octadecanoid acid (fatty acids), and Benzylmalonic acid (malonates). Additionally, high concentrations of tetracosane compounds (alkalenes) were detected in PRM. The main chemical constituents that were isolated and identified from PR included amides, alkaloids, phenylpropanoids, alkyl glycosides, and lignans (19), while Piperidine was found in leaves (20). Tetracosane is a straight-chain alkane containing 24 carbon atoms, playing a role as a plant metabolite and a volatile oil component. Tetracosane is a natural product found in *Cryptotermes brevis, Erucaria* *microcarpa*, and other organisms (PubChem), which has been explored in the pharmaceutical field. According to previous research (21), tetracosane is a potential bioactive compounds, providing a rationale for its traditional use in peptic ulcer treatment.

Specific compounds had also been found in PB, both in mature (PBM) and old leaves (PBO), including germacrene A (sesquiterpenoids), eugenol (terpenoids), and chaviol (phenols). These compounds were responsible for the distinctive aroma of PB, (22) which occurred due to the presence of oils, including terpenes and phenols. In this research, it was also discovered that the compounds beta.-selinene (sesquiterpenoids), delta.-Guaiene (sesquiterpenoids), and Linolelaidic acid (fatty acids) were only found in PBM at high concentrations, producing more essential oil compared to PBO. The main constituent of leaves is an oil with a chemical composition depending on the location observed, namely asbetle oil. Leaves produce compounds such as hydroxychavicol acetate, allylpyrocatechol, chavibetol, piperbetol, methylpiperbetol, piperol A and B. Hydrocydroxychavicol and eugenol, including phenolic compounds, consist of monocyclic fragrant ring with an alcoholic, aldehydic, or carboxylic group, which are essential in PB leaves, contributing to several bioactivities. Chavibetol is the main component of the essential oil, characterized by a highly spiced odor. Hydroxychavicol has also shown beneficial bioactivities anticarcinogenic and antimutagenic activities (22).

In PA, specific compounds were detected, namely Dilapiole and Hexadecane, which belong to the alkanes group. According to previous research (23), PA leaves and fruit contain 0.30% and 0.33% essential oil, respectively. Apiol was the most abundant chemical compound obtained in the essential oil of leaves and fruit, with concentrations of 57.10% and 66.31%, respectively. This essential oil successfully inhibited the growth of *Aspergillus niger* and *Cladosporium* sp. but was unable to inhibit *Fusarium oxysporum* and *Fusarium solani*. In this research, Germacene D was also detected, a class of sesquiterpenes compounds. Similarly, it was discovered (24) that based on gas chromatography analysis, 17.16% of Germacrene D was detected in leaves. This compound also suppressed the growth of lung cancer and leukemia cells in vitro.

Apart from specific compounds that are found in one species, 8,11,14-Eicosatrie, Trans-stigmasta-5, Dotriacontane, Tetratetracontane, Hexatriacontane, Delta-tochopherol, and Hexadecanoic acid, were also found in all species. These compounds were only found in PA, PC, and PB, due to a separate grouping in PR compared to the other three *Piper* species.

Conclusion

In conclusion, based on GC-MS results, a total of 40 secondary metabolite compounds were detected in four *Piper* species. PCA score plot analysis showed that there was a significant grouping of the compounds, where PC was grouped separately on the right, while other species were on the left. Furthermore, there were several specific compounds found in one species and not in others. Similar to mature and old leaves, some compounds were only found in one of these developmental phases. Alkaloid content contributed 25% of the total compounds detected, while fatty acids had the largest portion of 27.5%.

Supplementary Data

Fig. 1. Results of GC-MS analysis.

Fig. 2. Determination results for each *Piper* species.

Fig. 3. Secondary metabolite data that was submitted in Metaboanalyst.

References

1. Potzernheim M, Bizzo HR, Agostini-Costa, Vieira RF, Carvalho-Cilva M, Gracindo LBAM,

et al. Chemical Characterization of Seven *Piper* Species (Piperaceae) from Federal District, Brazil, based on Volatile Oil Constituents. Rev Bras Plantas Med. 2006;8:10–2.

2. Quijano-Abril MA, Callejas-Posada R, Miranda-Esquivel DR. Areas of endemism and distribution patterns for Neotropical *Piper* species (Piperaceae). J Biogeogr. 2006;33(7):1266–78.

3. Bhalerao S a, Verma DR, Gavankar R V, Teli NC, Rane YY, Didwana VS, et al. Phytochemistry, Pharmacological Profile and Therapeutic Uses of *Piper Betle* Linn. – An Overview. J Pharmacogn Phytochem Phytochem. 2013;1(2):10–9.

4. Article R, Dhanalakshmi D, Umamaheswari S, Balaji D, Santhanalakshmi R, Kavimani S. Phytochemistry and Pharmacology of *Piper Longum* –. 2017;6(1):381–98.

5. Dhurjad L, Sagle D, Deshmukh A, Narkhede M. A Review: Traditional Use, Phytochemical and Pharmacological Review of Red Betel Leaves (*Piper Crocatum* Ruiz & Pav). Asian J Pharm Res Dev. 2020;8(3):92–6.

6. P. Morais V, C. Fernandes C, H. G. Martins C, E. M. Crott A, L. D. Miranda M. Bioactive Hexane Extracts from *Piper aduncum* and *Xylopia aromatica* Against Bacterial Strains which Cause Food Poisoning. Rev Virtual Química. 2023;(June).

7. Macbride JF. Flora of peru Vol. XIII. 1893; Field Museum Press, Chicago.

8. Kale RN, Patil RY. High Performance Thin Layer Chromatography Fingerprinting Analysis of *Piper betle* L. Leaves. J Pharm Res Int. 2021;(February 2021):8–15.

9. Durant-Archibold AA, Santana AI, Gupta MP. Ethnomedical uses and pharmacological activities of most prevalent species of genus *Piper* in Panama: A review. J Ethnopharmacol [Internet]. 2018;217(January):63–82. Available from: https://doi.org/10.1016/j.jep.2018.02.008

10. Vermerris W, Ralph N. Phenolic Compound Biochemistry. In: Springer, Dordrecht. 1994. p. 211–34.

11. Barbosa-Filho1 JM, * MRP, Moura1 MD, Silva1 MS, Karla, Lima1 VB, et al. Anti-infl ammatory activity of alkaloids: A twenty-century review. Brazilian J Pharmacogn. 2006;16(1):109–39.

12. Cai YZ, Mei Sun, Jie Xing, Luo Q, Corke H. Structure-radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. Life Sci. 2006;78(25):2872–88.

13. Othman L, Sleiman A, Abdel-Massih RM. Antimicrobial activity of polyphenols and alkaloids in middle eastern plants. Front Microbiol. 2019;10(MAY).

14. Ernst M, Silva DB, Silva RR, Vêncio RZN, Lopes NP. Mass spectrometry in plant metabolomics strategies: From analytical platforms to data acquisition and processing. Nat Prod Rep. 2014;31(6):784–806.

15. Junairiah, Nurhariyati T, Marisan M, Suhargo L, Zuraidassanaaz NI. Extraction, Isolation and Characterization of Bioactive Compounds from Ethanol and Chloroform Extracts of P*iper sarmentosum* Roxb. Leaves. Asian J Plant Sci. 2023;22(2):290–4.

16. Salehi B, Zakaria ZA, Gyawali R, Ibrahim SA, Rajkovic J, Shinwari ZK, et al. *Piper* species: A comprehensive review on their phytochemistry, biological activities and applications. Vol. 24, Molecules. 2019.

17. Saccenti E, Hoefsloot HCJ, Smilde AK, Westerhuis JA, Hendriks MMWB. Reflections on

univariate and multivariate analysis of metabolomics data. Metabolomics. 2014;10(3):361–74.

18. Puspita PJ, Safithri M, Sugiharti NP. Antibacterial Activities of Sirih Merah (*Piper crocatum*) Leaf Extracts. Curr Biochem. 2019;5(3):1–10.

19. Salleh WMNHW, Ahmad F. Phytopharmacological investigations of *Piper retrofractum* vahl. – A review. Agric Conspec Sci. 2020;85(3):193–202.

20. Luyen BTT, Tai BH, Thao NP, Yang SY, Cuong NM, Kwon YI, et al. A new phenylpropanoid and an alkylglycoside from *Piper retrofractum* leaves with their antioxidant and α-glucosidase inhibitory activity. Bioorganic Med Chem Lett. 2014;24(17):4120–4. Available from: http://dx.doi.org/10.1016/j.bmcl.2014.07.057

21. Uddin SJ, Grice D, Tiralongo E. Evaluation of cytotoxic activity of patriscabratine, tetracosane and various flavonoids isolated from the Bangladeshi medicinal plant *Acrostichum aureum*. Pharm Biol. 2012;50(10):1276–80.

22. Nerkar VK, Lal PI, Chaudhary PH, Ruikar DB. A Comprehensive Review on *Piper Betel* Linn. International Journal of Pharmaceutical Research and Applications. 2023;8(2):1726-1734.

23. Wibawa IPAH, Saraswaty V, Kuswantoro F, Andila PS, Wardhani PK, Tirta IG, et al. A study of essential oil from an invasive *Piper aduncum* L. J Biol Udayana. 2019;23(2):50-58.

24. Mayanga-Herrera A, Tapia-Rojas S, Fukusaki-Yoshizawa A, Marcelo-Rodríguez Á, Amiel-Pérez J. Actividad citotóxica de la fracción clorofórmica de Piper aduncum y su efecto en el ciclo celular en líneas celulares de cáncer gástrico. Rev Peru Med Exp Salud Publica. 2020;37(3):471–7.

Secondary Metabolite Profile in Mature and Old Leaves of Four Piper Species

ORIGINALITY REPORT

20%	16%	8%	5%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	www.ajprd.com Internet Source	3%
2	ojs.unud.ac.id Internet Source	2%
3	Luli Zhou, Dingfa Wang, Hanlin Zhou. "Metabolic profiling of two medicinal Piper species", South African Journal of Botany, 2021 Publication	1%
4	lipi.go.id Internet Source	1%
5	www.rroij.com Internet Source	1%
6	www.phytojournal.com Internet Source	1%
7	repository.unej.ac.id Internet Source	1%
8	www.journaljpri.com Internet Source	

		1%
9	www.science.gov Internet Source	1%
10	Submitted to Jawaharlal Nehru University (JNU) Student Paper	1%
11	Submitted to Academic Library Consortium Student Paper	1%
12	academicjournals.org Internet Source	1%
13	hrcak.srce.hr Internet Source	1%
14	Ika Oktavianawati, Mardi Santoso, Sri Fatmawati. "Metabolite profiling of Borneo's Gonystylus bancanus through comprehensive extraction from various polarity of solvents", Scientific Reports, 2023 Publication	1%
15	Submitted to Universiti Malaysia Terengganu UMT Student Paper	<1%
16	Submitted to Taylor's Education Group Student Paper	<1%
17	riquim.fq.edu.uy Internet Source	<1%

18	www.chnfood.cn Internet Source	<1 %
19	www.acep.org Internet Source	<1 %
20	Shaikh Jamal Uddin, Darren Grice, Evelin Tiralongo. " Evaluation of cytotoxic activity of patriscabratine, tetracosane and various flavonoids isolated from the Bangladeshi medicinal plant ", Pharmaceutical Biology, 2012 Publication	<1 %
21	"Anti-Viral Metabolites from Medicinal Plants", Springer Science and Business Media LLC, 2024 Publication	<1 %
22	"Plant-derived Bioactives", Springer Science and Business Media LLC, 2020 Publication	<1 %
23	chimie.unibuc.ro Internet Source	<1 %
24	tjnpr.org Internet Source	<1 %
25	www.omicsonline.org Internet Source	<1 %